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(54) Tumor suppressor fusion proteins.

(57) The present invention provides a soluble molecular complex for introducing a cancer suppressor transcription factor to a mammalian cell, the complex comprising a fusion of a ligand for a mammalian cell membrane receptor releasibly bound to the cancer suppressor transcription factor. Further provided by the present invention are methods of treating pathologic conditions caused by gene dysfunction or alteration of gene products.

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small cell lung carcinoma cell line in vitro.

Figure 13 demonstrates that $p110^{RB-1125}$ localizes to the nucleus of H596 cells in a time dependent manner. (Radke, K. et al., "Membrane Association of 36,000 Dalton substrate for tyrosine phosphorylation in Chicken Embryo Fibroblasts transformed by avian sarcoma virus", Cell Biol., 97:1601-1611 (1983)).

The present invention provides a soluble molecular complex for introducing a tumor or cancer suppressor transcription factor to a mammalian cell. As used herein, the term "tumor or cancer suppressor transcription factor" refers to proteins that act to suppress cancer or tumor growth such as Rb or p53. It is intended that the term refers to both the full native proteins or to fragments modifications or derivatives which retain the tumor suppressor activity of the native protein. It is also intended that the term encompass proteins having a nuclear localization signal, native or synthetic. For example, the nuclear localization signal present on p56 RB is sufficient to direct nuclear translocation.

This invention provides ligands to a cell membrane receptor, that will induce endocytosis and carry-in the tumor suppressor transcription factor. The ligand must be releasibly bound to the tumor suppressor transcription factor, allowing escape of the tumor suppressor transcription factor into the cytoplasm ol the cell. As used herein the term "releasibly bound" is intended to encompass an extracellularly stable bond that is cleavable under appropriate conditions within the cell so as to release the tumor suppressor transcription factor intracellularly. For example, cleavage of the bond can be pH or enzymologically regulated. As used herein "ligand to a cell membrane receptor" refers to a molecule which binds to receptors located on the cell surface. Such receptors can be tissue specific or can be ubiquitously found on many or all cell surfaces. The cell membrane receptors all provide a -COOH binding site for the ligand. Examples of such receptors are GIP colon cell receptor, bombasin growth factor receptor on lung cells and ferretin.

Two ligands have been examined: Pseudomonas exotoxin A (PE) and interferon alpha (Wick, M.J. et al., "Analysis of the Structure-function relationship of Pseudomonas aeruginosa exotoxin A", Mol. Micro., 4:527-535 (1990); Rubinstein, M. et al., "The interferon receptors", Crit. Rev. Biochem., 21:249-275 (1986); Wileman, T. et al., "Receptor-mediated endocytosis", Biochem., 232(1):1-14 (1985); Yonehara, S. et al., "Cell surface receptor-mediated internalization of interferon: its relation to the antiviral activity of interferon", J. Gen. Virol., 64:2409-2418 (1983); Arnheiter, H. et al., "Microinjection of anti-interferon antibodies into cells does not inhibit the induction of an antiviral state by interferon", J.Virol., 52(1):284-07 (1984)). Domain 1 of exotoxin A is important to cell surface recognition and receptor binding while domain 2 is required for translocation out of the endosome (Liu, P.V. "The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. 3. Identity of the lethal toxins produced in vitro and in vivo", Infect. Dis., 116(4):481-9 (1966)). The fusion proteins have been constructed containing either partial domain 1, intact domain 1 or both domain 1 and 2. Both ligands, PE and interferon, were constructed as fusions with N-terminal truncated p56Rb and produced E. coli. It is significant that N-terminal truncated $p56^{RB}$ has no activity on its own, unlike $p110^{RB}$, nor is $p56^{RB}$ active when co-incubated with tumor cells. The fusion proteins so constructed were purified and tested. Unlike $p56^{RB}$ unmodified, the $p56^{RB}$ fusion proteins have the T-antigen binding activity and tumor growth suppression function of the full length $p110^{RB}$.In this invention, it is also shown that full length (unmodified) RB protein has growth suppression activity under pharmacologically acceptable conditions.

Exotoxin A of Pseudomonas aeruginosa is one member of a family of secreted bacterial toxins that are capable of covalently modifying specific target proteins within mammalian cells. Analysis of the identified domains show that the amino-terminal domain (domain 1) is involved in recognition of eukaryotic target cells. The central domain (domain 2) is involved in secretion of exotoxin A into the periplasm of Escherichia coli. Domain 2 also functions in translocation of exotoxin A from the eukaryotic endosome which containing the toxin after being internalized into susceptible eukaryotic cells via receptor-mediated endocytosis. The carboxy-terminal portion of exotoxin A (domain 3) encodes the enzymatic (toxin) activity of the molecule.

Exotoxin A enters eukaryotic cells via receptor-mediated endocytosis, is internalized into clathrin-coated pits, and proceeds into endosomes (Saelinger, C.B. et al., "Intracellular trafficking of Pseudomonas exotoxin A." Antibiot. Chemo., 39:149-59 (1987)). The receptor of exotoxin A is species-specific, and certain glycosphingolipids - asialo-GM1 and asialo-GM2, but not GM2, GM2, GM3, may act as receptors on the cell surface (Krivan, H.C. et al., "Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids" Proc. Natl. Acad. Sci. USA, 85(16):6157-61 (1988)). Using the characteristics of exotoxin A, three different fusion proteins were constructed see Example I and Figure 1 (PEA56RBD0 and PEA56RBD1), Figure 2 (PEA56RBD2S). Both PJH8, PJH14 and Pseudomonas aeruginosa strains are from ATCC (Rockville, MD). Plasmid 44-2 and E. coli BL21 (with pLysS) were provided by Dr. Wen Hwa Lee. Plasmid pflag (IBI, CT) and DH5 alpha competent cells (BRL, Bethesda, MD) were obtained from suppliers. Figure 3 summarizes the final structures of the different PE

(6). 3' primer for Pseudomonas aeruginosa DNA with Hind III site 5' AAGAAAGCTTTGCCGTCGCCGAG-GAACTC

(7). 3' primer for PJH14 with Ava I site 5' AATTCTCGGGAAAGTGCAGGCGATGAC 3'

(8). 5' primer for pCGS261 with Hind III site 5' AGCTCTAAGCTTTGTGATCTGCCTCAGACTC 3'

(9). 3' primer for pCGS261 with Ava I site 5' AATTCTCGGGTTCCTTACTCTTCAATCT 3'

PCR reactions were performed under the following conditions:

dNTP 10mM	8 µl;
buffer 10x	100 ng
5' primer	300 nM
3' primer	300 nM
formamide	1.5% volume
Taq Polymerase	1 unit
dH20 bring to	50 µl
mineral oil	50 µl

Reaction conditions were as follows:

95° C x 5'

94° C x 1'-----55° C x 1'-----72° C x 1-2' for 30 cycles

72° C x 10'

EXAMPLE II

Expression and Purification of Fusion Proteins PEA56RBD0, D1, D2S, FID56RB

Based upon the BL21(DE3)pLysS host organism of Rosenberg (Rosenberg, A.H. et al., "Vectors for selective expression of clone DNAs by T7 RNA polymerase", Gene, 56(1):125-35 (1987)), after transformation with the PEA56RBD0, D1, D2S plasmid, protein production could be induced by isopropyl-β-D-thiogalactopyranoside. For FID56RB, DH5 alpha was used as the host. Bacterial overnight cultures were prepared by direct inoculation of bacteria from master seed banks into LB media containing 100 µg/mL ampicillin and 20 µg/mL chloramphenicol. After growing 15 hours at 30°C in a New Brunswick Scientific shaker, an OD_{600} of between 2 to 3 was attained, the whole content of the seed flask was used to inoculate 3.4 L medium in a 5 L fermentor. The temperature of the fermentor was set at 28°C, the pH at 6.9, aeration at 0.5 L/min and agitation at 300 rpm. IPTG induction (0.2 mM final concentration) was performed when OD_{600} of the culture reached 7 to 8 and additional LB media and glucose were supplemented. Bacteria were harvested 3 hours after induction, pelleted and frozen at -80°C until use.

Cell homogenates were prepared using a microfluidizer model M110T at 10,000 psig in the lysis buffer containing 10mM phosphate, 1mM EDTA, and 1mM PMSF at pH 7.5. The resulting microfluidized cell lysate was spun down at 10,000 rpm in a JA-10 rotor for 25 minutes at 4°C. Western blot analysis utilizing ∞-Rb monoclonal antibody 318 of the fusion proteins indicating that they each have the correct molecular weight and react specifically with the Rb moiety at their C-termini is shown in Figure 7.

EXAMPLE III

Purification & Biological Activity of the D0 & D1 Fusion Proteins

For D0 and D1, the pellet was resuspended in 2X volumes (in mL) with respect to the wet cell weight (in grams) of lysis buffer and spun down again in the JA-10 rotor for 25 minutes. Hence, 2X volumes refers to this volume of lysis buffer. This washing procedure was repeated three times. The remaining pellet was stirred in 2X volumes of lysis buffer containing 0.1% Tween-80 at 4°C for 1 1/2 - 2 hours and then centrifuged again in the JA-10 rotor.

Following the 0.1% Tween-80 wash step, the pellet was resuspended in 2X volumes of lysis buffer containing 4M urea. This mixture was stirred for at least 1 1/2 - hours at 4°C, followed by centrifugation in the JA-10 rotor as before.

The pellet was resuspended in 2X volumes of lysis buffer containing 8M urea and stirred for at least 1 1/2 - 2 hours at 4°C. The resuspended material was centrifuged again as before, the supernatant contains the partially purified fusion proteins.

3. The soluble molecular complex of claim 2, wherein the RB agonist comprises a small molecule derivative of $p110^{RB}$.

4. The soluble molecular complex of claim 3, wherein the small molecule derivative of $p110^{RB}$ exhibits the biological activity of a soluble full-length retinoblastoma protein.

5. The soluble molecular complex of claim 4, wherein the biological activity includes a nuclear localization signal.

6. The soluble molecular complex of claim 4, wherein the biological activity comprises tumor growth suppression.

7. The soluble molecular complex of claim 3, wherein the small molecule derivative is $p56^{RB}$ and fragments thereof.

8. The soluble molecular complex according to any one of claims 1 to 7 wherein the ligand specifically binds to a cell membrane -COOH receptor or to asialoglycoprotein.

9. The soluble molecular complex of claim 8, wherein the asialoglycoprotein is asialo-GM1 or asialo-GM2.

10. The soluble molecular complex according to any one of claims 1 to 9, wherein the ligand is Pseudomonas exotoxin A or interferon alpha.

11. The soluble molecular complex according to any one of claims 1 to 10 whereby the fusion protein is covalently modified with a hydrophobic monomer.

12. A soluble molecular complex for introducing a cancer suppressor transcription factor to a mammalian cell selected from the group consisting of PEA56RBD0, PEA56RBD1, PEA56RBD2S and FID56RB.

13. A pharmaceutical composition comprising the soluble molecular complex according to any one of claims 1 to 12 and a pharmaceutically acceptable carrier.

14. The pharmaceutical composition of claim 13 for the treatment of retinoblastoma, bladder carcinoma, breast cancer, chronic myelogenous leukemia, acute myelogenous leukemia, testicular tumors, dysplastic and cancerous ulcerative colitis, sporadic sarcomas, prostate carcinoma, osteosarcoma, small-cell lung cancer, synovial sarcoma and other malignancies or hyperproliferative diseases characterized by RB gene dysfunction or alteration of the RB gene product.

15. A method of modifying a cell-regulatory activity of the Rb gene comprising contacting the cell with the soluble molecular complex according to any one of claims 1 to 12, thereby modifying the cell-regulatory activity.

16. The method of claim 15, wherein the ligand releases the RB agonist into the cytoplasm of the mammalian cell.

17. The method of claim 15 or 16, wherein the contacting is effected in vitro.

18. The method according to any one of claims 15 to 17, wherein the regulatory activity comprises tumor growth suppression.

Figure 2
pEA56Rb D_2S Construction

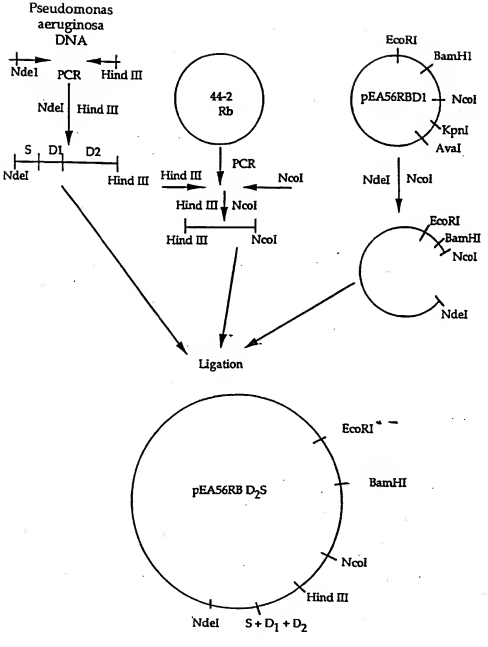

FIGURE 4

Ligand -Rb Fusion proteins: How do they work?

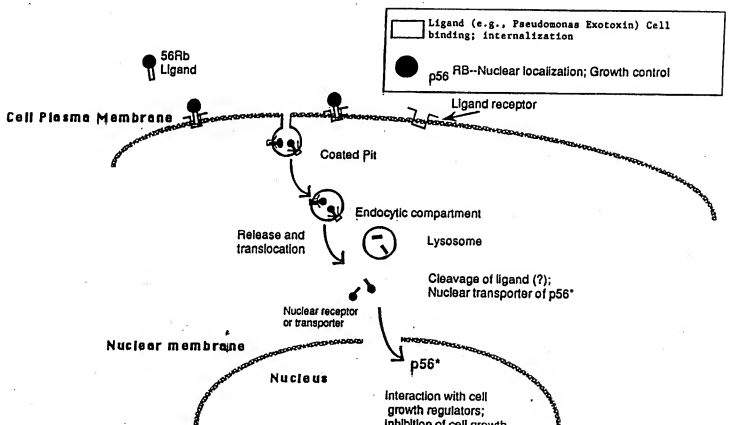

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FIGURE 6

Construction of an Interferon-Rb Fusion Protein

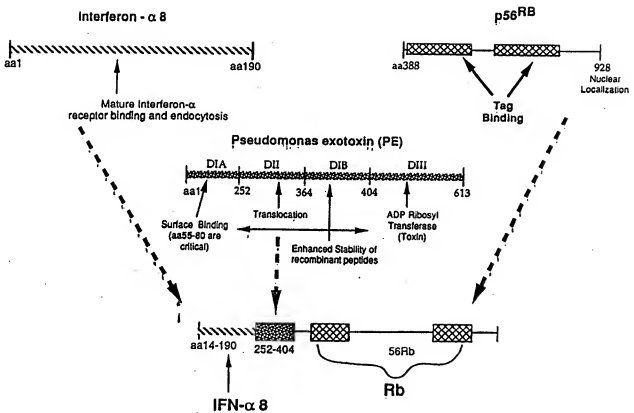

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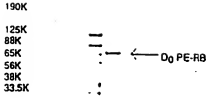
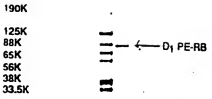
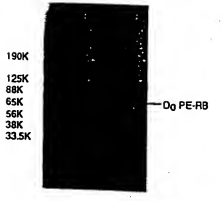
FIGURE 8



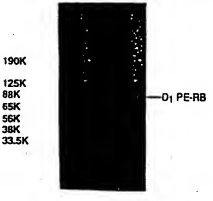
Panel A. SDS-PAGE gel of partially purified D_0 PE-RB Fusion Protein.
Lane 1 Molecular Weight Stds.
Lane 2 D_0 PE-RB



Panel B. SDS-PAGE gel of partially purified D_1 PE-RB Fusion Protein.
Lane 1 Molecular Weight Stds.
Lane 2 D_1 PE-RB



Panel C. Western Blot of partially purified D_0 PE-RB Fusion Protein.
Lane 1 Molecular Weight Stds.
Lane 2 D_0 PE-RB



Panel D. Western Blot of partially purified D_1 PE-RB Fusion Protein.
Lane 1 Molecular Weight Stds.
Lane 2 D_1 PE-RB

FIGURE 10

Normal p110^{RB} Produced in Baculovirus Inhibits the Growth of Rb Negative H128 SCLC Tumor Cells

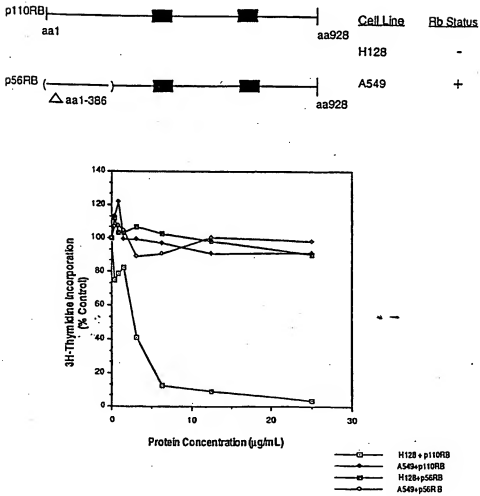
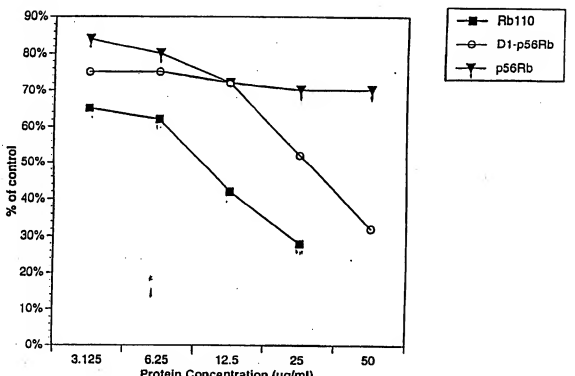


FIGURE 12

Growth Inhibition of Non-small Cell Lung Carcinoma NCI-H596 by Ligand-Fusion Protein D1-p56Rb



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PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 94 10 8445

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y	EP-A-0 529 160 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) * claims * * page 2, line 58 - page 3, line 2 * ---	1-6, 12-18	C07K19/00 A61K38/17 //C12N15/62
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol.87, no.12, June 1990, WASHINGTON DC, USA pages 4697 - 4701 D. HEIMBROOK ET AL. 'Transforming growth factor alpha-Pseudomonas exotoxin fusion protein prolongs survival of nude mice bearing tumor xenografts.' * abstract * ---	1-6, 12-18	
A	US-A-4 942 123 (LEE ET AL.) * example 1 * ---	1-6, 12-18	
A	WO-A-94 06910 (CANJI INC.) * claims * --- -/--	1-18	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C07K A61K

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
THE HAGUE	18 November 1994	Nooij, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
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D : document cited in the application
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& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

Remark: Although claims 15,16 and 18 are directed to a method of treatment of the human/animal body (Art. 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/composition